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THE FREE AMINO GROUPS OF CITRATE-EXTRACTED COLLAGEN

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The availability of the reactive groups of collagens is of special interest since the structural factors which regulate the solubility are thus far unknown. This report deals with the reactivity of the amino groups of citrate-extracted collagens with 2,4-dinitrofluorobenzene (DNFB). The free amino groups of collagen and its modifications were studied by Bowes & Moss (1951). These authors extended later (1953) their experiments to embrace also the citrate-extracted collagen (referred in this report as »procollagen»). Qualitatively, at least, the results reported here agree with theirs.

EXPERIMENTAL

Reagents. — The DNFB was supplied by Eastman & Co. and by Hoffman-La Roche. The chemicals were of the analytical grade except those used in chromatography, where *tert.* amyl alcohol of practical (Eastman) or technical (B.D.H.) grade was used. In the latter case the commercial product was distilled and the fraction with b.p. + 101 — 103°C used.

References. — The 2,4-dinitrophenyl (DNP-) derivatives were prepared according to the instructions of Sanger (1945) and Porter & Sanger (1948) from the following amino acids (from H & M, Santa Monica, Calif.): alanine, valine, leucine, *iso*leucine, serine, threonine, methionine, aspartic acid, glutamic acid, phenylalanine, tyrosine, proline, hydroxyproline, glycine, cysteine, arginine, histidine (the bis-DNP-derivative) and lysine (both the bis-DNP-lysine and the ε-DNP-lysine). Most of them were crystallised. A derivative was also prepared from d-glucosamine and crystallised from ethanol. An attempt to prepare hydroxylysine following the instruction of Robson & Selim (1953) resulted in a mixture of lysine and hydroxylysine, which was treated with DNFB after inactivation of the α-amino group as copper complex according to Sanger. When the resulting DNP-compounds

were chromatographed (see below) two spots were detected, one of ε-DNP-lysine and another, the ε-DNP-hydroxylysine, migrating closely behind.

Paper Chromatography of the DNP-compounds. — The principle was adopted from Blackburn & Lowther (1951). The solvents were saturated with pH 6.0 phthalate buffer (.05—.10 *M*) and the paper (Whatman No. 1) was sprayed with the buffer solution and air dried. All the chromatograms were run in the descending system.

The Rf-values of the DNP-amino acids were not constant, depending on the acidity of the original spot and on the temperature. Therefore appropriate mixed references were included in all the chromatograms. Some Rf-values may be quoted as examples.

DNP-aspartic acid	.05
DNP-glutamic acid	.08
DNP-serine	.40
DNP-hydroxyproline	.44
DNP-cysteine (bis-)	.44
DNP-glycine	.46
DNP-threonine	.57
DNP-proline	.60
DNP-alanine	.69
DNP-methionine	.79
DNP-valine	.83
DNP-leucine	.85
DNP-*iso*leucine	.85
DNP-phenylalanine	.88
DNP-tyrosine	.93
DNP-glucosamine	.95
bis-DNP-lysine	.70 (tailing)

With the Rf-value of about .70, between DNP-alanine and DNP-methionine, migrates the dinitrophenol, which fades in hydrochloric acid vapours. Dinitroaniline migrates near the front. The DNP-arginine migrates about .10 units ahead of ε-DNP-lysine, approximatively with bis-DNP-histidine. The ε-DNP-hydroxylysine seemingly migrates closely behind the corresponding lysine.

Using *tert.* amyl alcohol it is difficult to resolve the combination of DNP-serine, DNP-glycine and DNP-hydroxyproline, as also the combination of DNP-alanine and DNP-proline, which can be identified by its specific absorption spectrum. The proline derivatives also have a red brim around the spots. When the acidity was increased some improvement was obtained in the resolution of the first-mentioned DNP-amino acids.

Quantitative Estimations. — The spots were cut off and eluted with 2—5 ml. of 1% sodium bicarbonate solution or (the ether-insoluble) with *N* hydrochloric acid. The paper pulp was centrifuged down and the colour measured with the Beckman spectrophotometer at wave length of 3500 Å. Sanger states that above 3000 Å the absorption curves of all the DNP-

amino acids are essentially similar, that Beer's law is valid in concentrations less than 50 μM and that the standard curves of the different DNP-amino acids at 3500 Å differ only slightly. To control the conditions I repeated the standard curves with twice crystallised DNP-valine and DNP-lysine. The densities obtained were only about 2% lower than those reported by Sanger. There is no information available on the standard curve of the ε-DNP-hydroxylysine and the corresponding values of lysine were used. If it is assumed that a colour with an optical density of .100 can be measured accurately, about .015 μM of DNP-amino acid can be determined.

Collagen Samples. — One sample, designated as I, was prepared by Dr. Gross from carp swim bladder tunics by repeated citrate extractions and subsequent dialysis against water and final lyophilisation. This preparation was dissolved into 10% calcium chloride solution. Another specimen (II) originated from rabbit skin, which was first extracted twice with .2 *M* disodium phosphate and thereafter with pH 3.0 McIlwaine's buffer. The buffer solution was dialysed against .01 *M* disodium phosphate solution and the precipitate collected and washed with water and with small amount of 90% ethanol. All the preparatory work was carried out in the cold. This preparation was not easily soluble into calcium chloride and therefore a solution in .2 *M* citrate buffer (pH 3) was neutralised to pH 6.5 with sodium carbonate, centrifuged and the clear, viscous supernatant used.

Preparation of DNP-procollagen. — To 7.5 ml. of 10% calcium chloride solution, containing 75 mg. of sample I, 0.5 ml. of DNFB was added in 7.5 ml. of ethanol. Sodium tetraborate was added to saturation and the mixture shaken for two hours at room temperature. To the mixture 100 ml. of 90% ethanol was added to precipitate the DNP-protein, which was collected as slimy mass. The other procollagen sample (II) was used as citrate-containing solution, pH 6.5. To 50 ml. of the dilute solution .7 ml. of DNFB was added in 20 ml. ethanol. A stringy precipitate formed and the mixture was shaken for two hours. The procollagen was collected after an addition of still 200 ml. of ethanol. In this case the protein was treated actually in precipitated form.

Chromatography of the DNP-procollagen Hydrolysates. — The hydrolysis was performed at + 100° C in 11.5 *N* hydrochloric acid for about 12 hours. The hydrolysates (conc. about 2%) were extracted with peroxide-free ether, the extract dried with desiccated sodium sulphate and evaporated. For chromatography the residue was dissolved in chloroform or ethanol and these solutions applied on the paper using micro-pipette. The DNP-compounds tend to decompose and therefore the solutions were not stored. An infrared radiator in evaporation caused a disastrous destruction. The ether-insoluble fraction was evaporated in vacuum and the residue dissolved in water for application on the paper.

During the hydrolysis of DNP-proteins some destruction always occurs. The correction for this loss is the most important source of error in accuracy.

Porter & Sanger recommend hydrolysis in the above described conditions and find that for example 75% of the DNP-aspartic and DNP-glutamic acids remain unchanged. This was checked with 1.5 *mM* solution of DNP-aspartic acid which was treated in these conditions. On the estimation after the chromatography 62% was found unchanged. The dinitroaniline was formed as the decomposition product. The hydrolysis was found complete by repeated treatment, where in additional 12 hours' treatment a 20% destruction of the DNP-aspartic acid was observed.

The corrections made were thus based on the data by Sanger, checking the conditions with DNP-aspartic acid. The destruction rate was not affected by the presence of proteins or carbohydrates, which was ascertained by a control test with DNP-glycine dissolved in synovial fluid.

Determination of the Protein in DNP-compound. — The calculation was based on the combined content of aspartic and glutamic acids in the DNP-protein. The total hydrolysate was first extracted with ether to remove the small amount of the DNP-amino acids. Sodium bicarbonate was added to saturation and an excess of DNFB dissolved in ethanol. After two hours of shaking the DNP-amino acids were extracted with ether and chromatographed. The combined total content of the aspartic and glutamic acid was now measured as their DNP-derivatives and the total content of DNP-procollagen calculated using the data by Bowes & Kenten (1948) for bow hide collagen. The determination cannot be easily made on the weight basis since the amount of the DNP-moiety is not exactly known.

RESULTS

TABLE 1

REACTIVE AMINO GROUPS IN PROCOLLAGEN (M.MOL./100 G.)

	Procollagen I	Procollagen II	Total content of the residue in collagen
Free α-amino groups			
aspartic acid	.12	.16	47
alanine	.10	trace	106
serine	trace	none	33
glycine	trace	none	350
threonine	trace	none	20
Free ε-amino groups			
lysine	27 (19)	30 (21)	31
hydroxylysine	27 (8)	30 (9)	8

The ratio hydroxylysine/lysine was determined in sample I to be 1: 2.2 and in gelatin 1: 2.6. The mean 1: 2.4 was used in calculating the values of each which are given in brackets. The total contents are according to Bowes & Kenten (1948).

The results are presented in the table I and are corrected for destruction. When the ether-insoluble DNP-compounds of the DNP-procollagen II were chromatographed the ε-DNP-lysines were detected in their due location but in addition two spots migrating slightly faster than the lysines. In the ultraviolet light these spots were quite dark. They were interpreted as derivatives of the lysines but the mechanism is not clear. They cannot be produced by the strong hydrochloric acid, which was checked by the treatment of the DNP-gelatin. The ratio DNP-hydroxylysine/DNP-lysine was measured in a DNP-gelatin sample to avoid this complicating factor.

DISCUSSION

Since the determination of the total procollagen is based on its content of aspartic and glutamic acids it would be essential that the compositions of the rabbit skin procollagen and swim bladder collagen do not differ from data obtained for the cow hide. The analyses on the rabbit skin procollagen (Bowes, 1954) do not show such differences that they would invalidate the determination of the skin sample. On the swim bladder collagen the question remains somewhat in doubt.

The free amino groups of collagen have been determined by Green, Ang & Lamb (1953) using acetylation in a mildly alkaline medium. Altogether 40 m.mol/100 g. were reached. On the other hand Bowes & Moss found that only about 60% of the total ε-amino groups of collagen reacted with DNFB. In explanation of this disagreement may be suggested that some of the ε-amino groups of lysine can be reached by the acetyl group but not by the bulky DNP-radical.

It is still uncertain what significance can be attached to the small amounts of the DNP-amino acids in the DNP-procollagen. It does not seem probable that these amino groups have marked influence on the solubility. Their presence can also be explained as the byproducts of the treatment. If the minimum average molecular sizes are calculated, they are in order of 800.000, which supports the view of the high molecular weight of the procollagen provided that the end groups are not masked and that the polypeptide chain is not cyclic.

SUMMARY

DNP-derivatives were prepared from soluble collagens both in dissolved and in precipitated state. After hydrolysis the DNP-amino acids were studied with paper chromatography. Very small amounts of DNP-aspartic acid and DNP-alanine were detected. About one third of the ε-amino groups of these collagens were not available to react with dinitrofluorobenzene.

Dr. Walter Bauer and Dr. Jerome Gross (Massachusetts General Hospital, Boston, Mass.) placed the necessary facilities generously at my disposal during the first phase of this work. They also helped with much friendly advice and valuable criticisms. I had the privilege to consult also Dr. Joane H. Bowes in August 1953. To my chief, Dr. P. E. Simola, my thanks are due for his interest and suggestions.

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